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I, JONNE YABSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. 2002951611 for a patent by THE BAKER MEDICAL RESEARCH INSTITUTE as filed on 24 September 2002.

WITNESS my hand this
Eighth day of October 2003

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PROVISIONAL SPECIFICATION

Invention Title: COLESTEROL EFFLUX AND USES THEREOF

Applicant: THE BAKER MEDICAL RESEARCH INSTITUTE

The invention is described in the following statement:

CHOLESTEROL EFFLUX AND USES THEREOF

The present invention relates generally to modifying cell metabolism particularly, metabolism of cholesterol and to methods of modifying efflux of cholesterol from cells. The invention also relates to methods of preventing and treating cholesterol related conditions such as atherosclerosis by modifying cholesterol metabolism and efflux from cells which may reduce the incidence of atherosclerosis by preventing accumulation of cholesterol in cells.

BACKGROUND

Atherosclerosis is a disease of the arteries characterised by the appearance of fatty lesions along the inner surface of a blood vessel wall, also known as atheromatous plaques. Contributing to the narrowing of the vessel lumen, the resultant constriction to smooth blood-flow ultimately deprives vital organs of their blood supply. Atheromatous plaques also have an increased propensity to rupture and become dislodged from the vessel wall, thus contributing to further end-organ damage by becoming lodged in arteries supplying the heart, causing myocardial infarction (heart attack); or the brain, causing stroke. The clinical and social significance of this disease is evident in the fact that half the annual mortality in Western society results from heart and blood-vessel diseases of which atherosclerosis is the primary cause. Understanding the underlying causes of atherosclerosis is thus vital in the design of effective treatment strategies in combating this alarming statistic.

A key event in the formation of the earliest atherosclerotic lesion, the fatty streak, is the influx of macrophages into the artery wall and their subsequent uptake of abnormal lipoprotein particles and cholesterol, forming what are often referred to as foam cells. Cholesterol, being a major constituent of the atherosclerotic plaque, has thus become the major focus of treatment strategies.

Being the major sterol in the human body, cholesterol has, as its primarily function, the structural integrity of cell membranes. It is also implicated in vital bodily functions, and synthesis of a number of steroid hormones, including

estrogen, progesterone and testosterone. In the liver, cholesterol is also the precursor of the bile acids which, when secreted into the intestine, aid in digestive processes.

Whilst important to human physiology, elevated levels of cholesterol in the blood, and the type of lipoproteins that transport it through the blood, clearly promote the onset and progression of atherosclerosis. However, current treatment strategies designed to lower cholesterol levels in the blood and in the cells, whether it be by dietary means or via lipid-lowering drugs, have been shown to reduce the risk of end-organ damage, but have had no impact on the regression of existing atherosclerotic plaques.

The limitations in presently available treatments as they relate to cholesterol-lowering therapies, highlight the need for novel therapeutic approaches in attenuating the progression, and ultimately in promoting the regression or preventing the establishment, of atherosclerotic lesions. One such approach is the elimination of cholesterol from foam cells, stabilizing them against lethal rupture, and more importantly, promoting the regression of existing lesions and a reversal of disease severity. The elimination of cholesterol from macrophage foam cells of atherosclerotic plaques, or cholesterol efflux, appears to be regulated by a number of factors, both intracellular and extracellular.

Accumulation of cholesterol is a result of an imbalance between pathways delivering cholesterol to cells and removing it. Pathways responsible for delivery of cholesterol to cells have been investigated and treatment based on this knowledge has been applied with great success. To achieve further progress in reducing atherosclerosis and consequently the risk of heart diseases and stroke, the pathway related to removing cholesterol from cells may also be targeted. This pathway is the reverse cholesterol transport (RCT) pathway.

The RCT pathway removes excess cholesterol from extrahepatic tissues including the vessel wall, thus preventing development of atherosclerosis. The first and most likely rate-limiting step of reverse cholesterol transport is

cholesterol efflux which is the transfer of cholesterol from cells to acceptors in plasma. Two pathways of cholesterol efflux are currently known. One involves lipidation of lipid-free or lipid-poor apolipoprotein A-I (apoA-I), and is most likely mediated by the ABCA1 transporter. The other involves transfer of cholesterol from plasma membrane caveolae to lipidated apoA-I or mature high density lipoprotein (HDL). Induction of ABCA1 and caveolin results in a stimulation of cholesterol efflux. However, control of these mechanisms of efflux remains unclear.

Cholesterol oxidation in the liver is a major pathway of cholesterol catabolism resulting in the conversion of cholesterol to bile acids. Oxidation of cholesterol in other cells such as macrophages and endothelial cells may also occur and is catalysed by the enzyme sterol-27 hydroxylase (CYP27) which converts cholesterol to 27 hydroxy cholesterol and 3-beta-hydroxy-5-cholestenoic acid. However, these oxidized forms of cholesterol may be toxic to the cells and are often released from the cells since the oxidized form of cholesterol is more hydrophilic . A build up of these byproducts is therefore undesirable for reasons of toxicity and deposition of these oxidized forms of cholesterol may lead to plaque formation, atherosclerosis and coronary heart disease. Whilst oxidation might be beneficial converting cholesterol to a form that can be easily released from the cell, the conversion must be regulated at a level that is non toxic or detrimental to the cell. Therefore, it would be desirable to increase cholesterol efflux without increasing toxic oxidized cholesterol byproducts.

It is an objective of the invention to enhance protection against accumulation of cholesterol, development of atherosclerosis and heart disease.

SUMMARY OF THE INVENTION

In a first aspect of the present invention, there is provided a method of modulating cholesterol efflux in a cell, said method comprising

modulating expression and/or activity of sterol 27-hydroxylase (CYP27) in the cell.

Applicants have found that cholesterol can be removed by cholesterol efflux which is regulated by CYP27. More surprisingly the cholesterol effluxed remains in the form of cholesterol and not as oxidized cholesterol as would be expected in the presence of CYP27.

In a further preferred aspect of the present invention, there is provided a method of modulating cholesterol efflux in a cell, said method comprising:

transfecting the cell with a gene encoding CYP27; and

modulating expression and/or activity of the transfected CYP27 in the cell.

This method of modulating cholesterol efflux by directly affecting expression of CYP27 is preferentially used to modulate CYP27 expression from the natural levels. Preferably, the introduction of a construct with the gene for CYP27 will increase the level of CYP27 upon expression and thereby affect the cholesterol efflux.

In a preferred aspect there is provided a method of increasing cholesterol efflux in a cell, said method comprising

increasing expression and/or activity of CYP27 in the cell.

In a further preferred aspect of the present invention, there is provided a method of increasing cholesterol efflux from a cell, said method comprising

transfecting the cell with a gene encoding CYP27; and

increasing expression of the transfected CYP27.

In another aspect of the present invention there is provided a cell with modulated cholesterol efflux, said cell having modulated CYP27 expression and/or activity.

Preferably the cell is transfected with a gene encoding CYP27. The cell is therefore capable of modulation, preferably having increased cholesterol efflux upon increased CYP27 expression.

In yet another aspect of the present invention, there is provided a method of treating a cholesterol related condition in a patient by modulating cholesterol efflux from a cell of the patient, said method comprising:

modulating expression and/or activity of CYP27 in the cell.

In a further preferred aspect of the present invention, there is provided a method of treating a cholesterol related condition in a patient by modulating cholesterol efflux in the patient, said method comprising:

introducing modulated cells to the patient, wherein said cells have modulated expression and/or activity of CYP27.

In yet another aspect of the present invention there is provided a method of identifying a compound which modulates cholesterol efflux in a cell, said method comprising:

contacting the compound to the cell;

detecting a change in CYP27 expression and/or activity in the cell relative to a cell which has not been contacted with the compound.

FIGURES

Figure 1 shows synthesis of 27-hydroxycholesterol in cells transfected with CYP27. CHOP cells were transfected with CYP27A1 or mock transfected with pcDNA1 for 48h. Following transfection, cells were incubated for 24h in serum-free medium containing [1-^{14}C] acetate. Cells were then washed and incubated for 2 h with 5% human plasma. Sterols from both cells and medium were extracted and separated with TLC and counted.

Figure 2 shows cholesterol efflux to human plasma and lipid-free apolipoprotein A-I. CHOP cells transfected with CYP27, mock-transfected and non-transfected were labeled with [^{3}H]cholesterol. Cells were then incubated with human plasma (final concentration 5%), human lipid-free apoA-I (final concentration 30 µg/ml) or serum-free medium alone for 2h at 37°C in a CO_2-incubator. Medium was collected, cells washed and the amount of radioactivity in the cells and medium determined on a β-counter. Cholesterol efflux is expressed as the percentage of labeled cholesterol moved from cells to medium (i.e. radioactivity

in the medium/radioactivity in the medium + radioactivity in the cells). Means ± SD of quadruplicate determinations are shown. *p<0.001 *versus* mock-transfected and nontransfected cells.

Figure 3 shows time-course (A) and dose-dependence (B) of cholesterol efflux to human plasma. CHOP cells transfected with CYP27 or mock-transfected were labeled with [^{3}H] cholesterol. Cells were then incubated with human plasma added at a final concentration of 5% (A) or at the indicated concentrations (B), or serum-free medium alone at the indicated periods of time (A) or for 2h (B) at 37°C in a CO_2-incubator. Medium was then collected, cells washed and the amount of radioactivity in the cells and medium determined in a β-counter. Cholesterol efflux is expressed as the percentage of labeled cholesterol moved from cells to medium (i.e. radioactivity in the medium/radioactivity in the medium + radioactivity in the cells). Background values (i.e. the efflux to the medium alone) were subtracted. Means ± SD of triplicate determinations are shown.

DESCRIPTION OF THE INVENTION

In a first aspect of the present invention, there is provided a method of modulating cholesterol efflux in a cell, said method comprising

modulating expression and/or activity of sterol 27-hydroxylase (CYP27) in the cell.

Sterol 27 hydroxylase (CYP27) is implicated in bile acid synthesis and is primarily found in hepatic cells. Hence its role is to convert cholesterol to bile acids in hepatic cells and thereby remove cholesterol from the cell in this manner. However, this enzyme is not commonly found in all cells and its role particularly in the macrophage and in epithelial cells is largely unknown. There is no requirement to produce bile in these cells and therefore the role of CYP27 is different. The enzyme has been found to be involved in removal of cholesterol from macrophages and smooth muscle cells and the removal of cholesterol is by oxidation of cholesterol to more effluxable forms of cholesterol such as 27 hydroxy-cholesterol and 3-beta-hydroxy-5-cholestenoic acid. However, Applicants have found that cholesterol can be removed by cholesterol

efflux which is regulated by CYP27. More surprisingly the cholesterol effluxed remains in the form of cholesterol and not as oxidized cholesterol as would be expected in the presence of CYP27.

In vessel walls, the reverse cholesterol transport pathway removes excess cholesterol, but the actual process of removal and the steps involved is complex. Applicants have found that cholesterol efflux from cells to acceptors such as apolipoprotein A-1 in plasma, mediated by sterol 27-hydroxylase, may contribute to the removal of cholesterol from the cells of the vessel wall and that the efflux of cholesterol is in an unoxidized form and is regulated by the enzyme CYP27.

The cell in which cholesterol efflux is modulated may be any cell that can produce cholesterol and can export cholesterol by cholesterol efflux. Preferred cells include, but are not limited to, hepatic cells, macrophages, endothelial cells, smooth muscle cells and other cells of the vessel wall. Most preferably the cells are from a vessel wall that is associated with an atherosclerotic plaque. However, it is also contemplated in the present invention to use stem cells that may differentiate to somatic cells in which cholesterol is effluxed. Preferably the somatic cell is selected from hepatic cells, macrophages, endothelial cells and other vessel cells; preferably, the stem cell is a haematopoietic stem cell.

Sterol-27-hydroxylase (CYP27) may be any form of the hydroxylase that oxidises cholesterol and converts cholesterol to 27 hydroxy cholesterol and 3-beta-hydroxy-5-cholestenoic acid. An "equivalent" as used herein is any compound that can behave and functions in a similar manner to CYP27 which may include various isoforms of the enzyme. The function of the enzyme may include its ability to oxidize cholesterol but also to contribute to any upstream or downstream reactions that can influence efflux of cholesterol

It is thought that the product of cholesterol oxidation by CYP27 regulates the efflux of un-oxidised cholesterol. It can either be a regulator of one of the genes involved in cholesterol efflux (or its regulation) or it can change the properties of the membranes (or a specific part of the membrane) to stimulate cholesterol

efflux. The activity of CYP27 in the cells is rather low (even after transfection) and although almost all oxidised cholesterol is released, it consists of only about 2-3% of total (i.e. oxidised plus non-oxidised) cholesterol released. Thus the role of CYP27 in cholesterol oxidation does not change.

Throughout the description and claims of this specification, the word "comprise" and variations of the word, such as "comprising" and "comprises", is not intended to exclude other additives, components, integers or steps.

The term "modulating" cholesterol efflux as used herein means to alter the rate of which cholesterol is removed from the cell. The degree of alteration is determined by comparing against natural rates of cholesterol efflux from the cell. "Modulating" includes adjusting cholesterol efflux to increase or decrease efflux. Preferably, the efflux is increased. Hence by targeting CYP27, cholesterol efflux can be manipulated.

Therefore, the discovery by the inventors that CYP27 is central to cholesterol efflux wherein the efflux is substantially in the form of unmodified cholesterol has provided a means by which cholesterol efflux can be changed, altered or adjusted as required. By "substantially" we mean predominantly in the form of non-oxidized cholesterol rather than the oxidized forms including 27 hydroxy cholesterol and 3-beta-hydroxy-5-cholestenoic acid. When cholesterol is effluxed, some is effluxed in this oxidized form. However, when regulated or modulated by CYP27, cholesterol in non-oxidized form is predominantly effluxed.

The amount of cholesterol effluxed from the cell will be dependent upon the conditions imposed on the cell. Preferably, 5 – 15% of cholesterol in the cell is released from the cell. However, these amounts may vary under different conditions. The form of cholesterol released may be in an oxidised or non-oxidised form. Preferably, of the 5 - 15%, approximately 0.1 – 0.3% may be released in an oxidised form and the remaining portion released as a non-oxidised form. However, since there is very little oxidised cholesterol in the cell (2-3%), the relatively small amount of oxidised cholesterol released may comprise about 80% of the amount of oxidised cholesterol formed by CYP27.

The term "modulating expression and/or activity of CYP27" as used herein includes modifying or altering the expression and/or activity of CYP27 compared to unmodified levels of CYP27. Expression and/or activity may be increased or decreased compared to unmodified levels to increase or decrease cholesterol efflux. Preferably, expression and/or activity of CYP27 is increased to increase cholesterol efflux.

Modulation of CYP27 expression and/or activity may be achieved by direct or indirect methods. Modulation of expression and/or activity of CYP27 may be achieved using direct methods known to those of skill in the art and include, but are not limited to, introduction of genes encoding CYP27, knockout technology, antisense technology, triple helix technology, targeted mutation, gene therapy and regulation by agents acting on transcription. Indirect methods for modulating expression and/or activity of CYP27 may include targeting upstream or downstream regulators.

"Activity" as used herein relates to a function of a CYP27 in a cell, and includes the ability of CYP27 and/or products of the reaction catalysed by CYP27 to bind to chaperone, or upstream or downstream effector molecules thereby activating or repressing upstream or downstream pathways which affect cholesterol efflux. The term "activity" also includes the ability of CYP27 to oxidize cholesterol.

In a further preferred aspect of the present invention, there is provided a method of modulating cholesterol efflux in a cell, said method comprising:

transfecting the cell with a gene encoding CYP27; and

modulating expression and/or activity of the transfected CYP27 in the cell.

This method of modulating cholesterol efflux by directly affecting expression of CYP27 is preferentially used to modulate CYP27 expression from the natural levels. Preferably, the introduction of a construct with the gene for CYP27 will increase the level of CYP27 upon expression and thereby affect the cholesterol efflux.

As discussed above, any cell can accept a gene encoding CYP27. However, ideally, the cell is a cell that requires efficient efflux of cholesterol. All cells efflux cholesterol, but cells of the vessel wall are most preferred and are those where this matters most because impaired cholesterol efflux in these cells leads to atherosclerosis. Stem cells are also considered within the scope of the present invention. These will be particularly useful for gene therapy and upon differentiation can be modulated in the appropriate regions of the body such as in vessel walls or in cardiac tissue to effectively cause cholesterol efflux upon further expression of CYP27.

The gene for CYP27 may be obtained from the Baker Medical Research Institute and inserted into a mammalian expression vector such as pcDNA3 to form a construct or vector that may be transfected into the cell to express CYP27.

Preferably, a gene sequence for CYP27 is operably linked to a regulatory sequence which is capable of providing for the expression of the coding sequence by a cell. The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

The term "regulatory sequences" includes promoters and enhancers and other expression regulation signals. These may be selected to be compatible with the cell for which the expression vector is designed. Mammalian promoters, such as β-actin promoters and the myosin light chain promoter may be used. However, other promoters may be adopted to achieve the same effect. These alternate promoters are generally familiar to the skilled addressee. Mammalian promoters also include the metallothionein promoter which can upregulate expression in response to heavy metals such as cadmium and is thus an inducible promoter. Tissue-specific promoters may be used. Viral promoters may also be used, for example the Moloney murine leukaemia virus long

terminal repeat (MMLV LTR), the promoter rous sarcoma virus (RSV) LTR promoter, the SV40 promoter, the human cytomegalovirus (CMV) IE promoter, herpes simplex virus promoters or adenovirus promoters. All these promoters are readily available in the art.

Such vectors may be transfected into a suitable cell in which cholesterol efflux is desired to provide for expression of a polypeptide encoding CYP27.

The vectors may be, for example, plasmid, virus or phage vectors provided with an origin of replication, optionally a promoter for the expression of the CYP27 and optionally a regulator of the promoter. The vectors may contain one or more selectable marker genes for example an ampicillin resistance gene in the case of a bacterial plasmid or a neomycin resistance gene for a mammalian vector. Vectors may be used *in vitro*, for example for the production of RNA or used to transfect or transform a host cell. The vector may also be adapted to be used *in vivo* for example in a method of gene therapy or a DNA vaccine. Most preferably the pcDNA3 vector is used. Preferably the vector contains the G418 (geneticin) resistance gene.

The cells in which the vector is transfected is expected to provide for such post-translational modifications (eg myristolation, glycosylation, truncation, lapidation and tyrosine, serine or threonine phosphorylation) as may be needed to confer optimal biological activity on recombinant expression products.

The vector may be transfected into the cell by any means available to the skilled addressee. Preferably, the vector is introduced by calcium phosphate precipitation, electroporation, biolistics, lipofection, DEAE Dextran or adenoviral or retroviral infection. However, this invention is not restricted to these methods.

The expression of CYP27 may be increased to a level above the normal CYP27 expression to favour and enhance cholesterol efflux. The degree of enhancement may be measured by actual cholesterol effluxed such as, but not

limited to measuring the release of labelled cholesterol when cellular cholesterol or a specific pool of it is labelled prior to the efflux experiment or measuring of cholesterol mass in cells and/or medium by commercially available fluorometric assays.

In a preferred aspect there is provided a method of increasing cholesterol efflux in a cell, said method comprising

increasing expression and/or activity of CYP27 in the cell.

By increasing the expression and/or activity of CYP27 in the cell, it has now been found that cholesterol efflux can be increased. The increase in cholesterol efflux is not due to increased efflux of oxidized cholesterol, but of mostly non-oxidized cholesterol. Quite unexpectedly, applicants have found that increasing CYP27 expression and/or activity does not lead to marked increases in oxidation products. The small amount of oxidized cholesterol is very low and not toxic at this concentration. High concentrations would generally be expected to be toxic. However, there is a marked increase in the efflux of non-oxidized cholesterol from the cell.

In a further preferred aspect of the present invention, there is provided a method of increasing cholesterol efflux from a cell, said method comprising

transfecting the cell with a gene encoding CYP27; and

increasing expression of the transfected CYP27.

Modulation of CYP27 to increase expression and/or activity may be achieved by inducing expression of CYP27 by transfection of a construct containing the CYP27 gene or by overexpressing the gene in the cell. By introduction of an exogenous CYP27 or a construct to express exogenous CYP27, the ability of CYP27 to increase cholesterol efflux may be modulated.

The cells are preferably transfected with CYP27 by any means that introduces a CYP27 gene to the cell. Preferably, the gene encoding CYP27 is transfected into the cell via an expression vector by methods available to the skilled addressee or as described above.

Preferably a construct of CYP27 is introduced or transfected into the cell to increase the expression of CYP27 thereby increasing cholesterol efflux. Increasing the expression may be achieved by any means known to the skilled addressee including the induction of promoters in the construct. Vectors may be used with regulatory regions that respond to tetracycline, mifepristone or ecdysone.

However, the expression and/or activity may also be increased by indirect methods of targeting indirect regulators to upregulate the gene. These regulators may act on the promoters that cause expression of the gene or they may act on upstream or downstream molecules that affect the enzyme. For instance adrenodoxin and adrenodoxin reductase may be targeted because they are important for the flow of electrons during the CYP27 enzymatic reaction. In transfection studies, fusion of all three proteins into a single chimera, or transfected in tendem, may generate more enzymatic activity than addition of the CYP27 gene alone. The chimera would be the molecule of choice for gene therapy work. It may be delivered in a vector with a promoter containing regulatory regions which preferably respond to metals, tetracycline, mifepristone or ecdysone. The chimera may also be delivered in tandem with a vector expressing another protein which has been shown to increase cholesterol efflux, such as, but not limited to apolipoprotien A1. The endogenous gene may be regulated by dexamethasone which increases CYP27 expression. Promoter studies show that CYP27 is upregulated by dexamethasone and downregulated by cyclosporin A and cholic acid. Accordingly, dexamethasone and cyclosporin A and cholic acid may be successfully used to regulate CYP27 expression.

Regulation of the gene expression may generally be achieved by the use of molecules reacting with the promoter of the gene or with a promoter of a nuclear factor regulating the gene, or by RNA processing including splicing and degradation. The activity of enzyme itself may also be targeted by phosphorylation, or allosteric regulation or regulation of the enzyme degradation such as by the use of protease inhibitors.

Increased expression and/or activity of CYP27 may be achieved by any means that can increase endogeneous CYP27 expression and/or activity thereby resulting in increased cholesterol efflux.

Preferably, the increased cholesterol efflux is via a mechanism which involves transfer of cholesterol from plasma membrane caveolae. Preferably, this method of cholesterol efflux induces a "fast" efflux of cholesterol by releasing cholesterol from more accessible sites of the plasma membrane. Accordingly, the present invention provides a method of increasing cholesterol efflux from caveolae, said method comprising:

increasing expression and/or activity of CYP27 in the cell.

More preferably, the increased expression and/or activity is induced by transfection of the cell with CYP27. The expression of the CYP27 is therefore increased to increase cholesterol efflux. Cholesterol efflux may also be increased further in this setting by cotransfection with a plasmid expressing apolipoprotein A1 or by introduction of apolipoprotein, as previously described.

Alternatively, modulation of CYP27 to decrease expression and/or activity in the cell may be achieved using antagonists, inhibitors, mimetics or derivatives of CYP27. The terms "antagonist" or "inhibitor", as used herein, refer to a molecule which, when bound to CYP27, blocks or modulates the activity of CYP27. Antagonists and inhibitors may include proteins, nucleic acids, carbohydrates, antibodies or any other molecules including ligands which bind to CYP27. Other modulators of the activity and/or expression of CYP27 include a range of rationally-designed, synthetic inhibitors.

Cholesterol efflux has been implicated as a potent regulator of cell growth: the less efflux the more growth and vice versa. Accordingly, decreases in cholesterol efflux may be useful in applications including cancer treatment for inhibition of growth by stimulating efflux or regeneration by stimulation of growth by inhibition of the efflux.

In another aspect of the present invention there is provided a cell with modulated cholesterol efflux, said cell having modulated CYP27 expression and/or activity.

Preferably the cell is transfected with a gene encoding CYP27. The cell is therefore capable of modulation, preferably having increased cholesterol efflux upon increased CYP27 expression.

The cell may be any cell that can efflux cholesterol. Preferably the cell is selected from the group including, but not limited to, hepatic cells, macrophages, vessel cells, endothelial cells, smooth muscle cells or stem cells. Preferably the stem cells are capable of differentiating to any of the cells selected from the group including hepatic cells, macrophages, endothelial cells and other vessel cells . Preferably, the stem cells are hematopoietic stem cells.

In yet another aspect of the present invention, there is provided a method of treating a cholesterol related condition in a patient by modulating cholesterol efflux from a cell of the patient, said method comprising:

modulating expression and/or activity of CYP27 in the cell.

The term "treatment" is used herein in its broadest sense to include preventative treatments or treatment that are intended to prevent further progression of the condition. In this respect, the treatment may include modulating CYP27 expression and/or activity at a level to prevent further progression of the cholesterol related condition by affecting the cholesterol efflux from the cell.

A "cholesterol related condition" as used herein may include any vascular conditions, including myocardial infarction, atherosclerosis, stroke, or peripheral vascular disease. Generally, these conditions result from an accumulation of cholesterol in plaques thereby causing narrowing of the vessel lumen in vessels, such as blood vessels, resulting in the condition which often manifests in tissue damage or death. Preferably the cholesterol related condition is atherosclerosis.

Preferably, the modulation of the expression and/or activity of CYP27 is increased to increase cholesterol efflux.

In an even further preferred aspect of the present invention, there is provided a method of treating a cholesterol related condition in a patient by modulating cholesterol efflux in a cell in the patient, said method comprising:

introducing a gene construct to modulate expression and/or activity of CYP27 in the cell in the patient.

The present invention also encompasses gene therapy whereby a gene encoding CYP27 and/or a CYP27 regulator, is regulated in a patient. Various methods of transferring or delivering DNA to cells for expression of the gene product protein, otherwise referred to as *gene therapy*, are disclosed in *Gene Transfer into Mammalian Somatic Cells* in vivo, N. Yang, Crit. Rev. Biotechn. 12(4): 335-356 (1992), which is hereby incorporated by reference.

Strategies for treating these medical problems with gene therapy include therapeutic strategies such as identifying a defective gene or protein and then adding a functional gene to either replace the function of the defective gene or to augment a slightly functional gene; or prophylactic strategies, such as adding a gene for the product protein that will treat the condition or that will make the tissue or organ more susceptible to a treatment regimen. As an example of a prophylactic strategy, a gene such as that for CYP27 and/or a CYP27 regulator may be placed in a patient and thus prevent occurrence of atherosclerosis or a related condition; or a gene that makes a cell more susceptible to modulation of cholestrol efflux.

Many protocols for transfer of CYP27 DNA, or CYP27 regulatory sequences are envisioned in this invention. Transfection of promoter sequences, or other sequences which would modulate the expression and/or activity of CYP27 are also envisioned as methods of gene therapy. An example of this technology is found in Transkaryotic Therapies, Inc., of Cambridge, Mass., using homologous recombination to insert a "genetic switch" that turns on an erythropoietin gene in

cells. See Genetic Engineering News, Apr. 15, 1994. Such "genetic switches" could be used to activate CYP27 (or CYP27 regulators) in a cell.

Gene transfer methods for gene therapy fall into three broad categories: physical (e.g., electroporation, direct gene transfer and particle bombardment), chemical (lipid-based carriers, or other non-viral vectors) and biological (virus-derived vector and receptor uptake). For example, non-viral vectors may be used which include liposomes coated with DNA. Such liposome/DNA complexes may be directly injected intravenously into the patient. Additionally, vectors or the "naked" DNA of the gene may be directly injected into the desired organ, tissue or tumor for targeted delivery of the therapeutic DNA.

Gene therapy methodologies can also be described by delivery site. Fundamental ways to deliver genes include *ex vivo* gene transfer, *in vivo* gene transfer, and *in vitro* gene transfer.

Chemical methods of gene therapy may involve a lipid based compound, not necessarily a liposome, to ferry the DNA across the cell membrane. Lipofectins or cytofectins, lipid-based positive ions that bind to negatively charged DNA, may be used to cross the cell membrane and provide the DNA into the interior of the cell. Another chemical method may include receptor-based endocytosis, which involves binding a specific ligand to a cell surface receptor and enveloping and transporting it across the cell membrane.

Many gene therapy methodologies employ viral vectors such as retrovirus vectors to insert genes into cells. A viral vector can be delivered directly to the *in vivo* site, by a catheter for example, thus allowing only certain areas to be infected by the virus, and providing long-term, site specific gene expression. *In vivo* gene transfer using retrovirus vectors has also been demonstrated in mammary tissue and hepatic tissue by injection of the altered virus into blood vessels leading to the organs.

Viral vectors may be selected from the group including, but are not limited to, retroviruses, other RNA viruses such as poliovirus or Sindbis virus, adenovirus,

adeno-associated virus, herpes viruses, SV 40, vaccinia and other DNA viruses. Replication-defective murine retroviral vectors are the most widely utilized gene transfer vectors and are preferred. Adenoviral vectors may be delivered bound to an antibody that is in turn bound to collagen coated stents.

Mechanical methods of DNA delivery may be employed and include, but are not limited to, fusogenic lipid vesicles such as liposomes or other vesicles for membrane fusion, lipid particles of DNA incorporating cationic lipid such as lipofectin, polylysine-mediated transfer of DNA, direct injection of DNA, such as microinjection of DNA into germ or somatic cells, pneumatically delivered DNA-coated particles, such as the gold particles used in a "gene gun," inorganic chemical approaches such as calcium phosphate transfection and plasmid DNA incorporated into polymer coated stents. Ligand-mediated gene therapy, may also be employed involving complexing the DNA with specific ligands to form ligand-DNA conjugates, to direct the DNA to a specific cell or tissue.

The DNA of the plasmid may or may not integrate into the genome of the cells. Non-integration of the transfected DNA would allow the transfection and expression of gene product proteins in terminally differentiated, non-proliferative tissues for a prolonged period of time without fear of mutational insertions, deletions, or alterations in the cellular or mitochondrial genome. Long-term, but not necessarily permanent, transfer of therapeutic genes into specific cells may provide treatments for genetic diseases or for prophylactic use. The DNA could be reinjected periodically to maintain the gene product level without mutations occurring in the genomes of the recipient cells. Non-integration of exogenous DNAs may allow for the presence of several different exogenous DNA constructs within one cell with all of the constructs expressing various gene products.

Gene regulation of CYP27 and/or CYP27 regulators may be accomplished by administering compounds that bind to CYP27 genes, or control regions associated with the CYP27 genes, or corresponding RNA transcript to modify the rate of transcription or translation. Additionally, cells transfected with a DNA sequence encoding CYP27 and/or CYP27 regulators may be administered to a

patient to provide an *in vivo* source of CYP27 and/or a CYP27 regulator. For example, cells may be transfected with a vector containing a nucleic acid sequence encoding CYP27 and/or a CYP27 regulator. The term "vector" as used herein means a carrier that can contain or associate with specific nucleic acid sequences, which functions to transport the specific nucleic acid sequences into a cell. Examples of vectors include plasmids and infective microorganisms such as viruses, or non-viral vectors such as ligand-DNA conjugates, liposomes, lipid-DNA complexes. It may be desirable that a recombinant DNA molecule comprising a CYP27 and/or CYP27 regulator DNA sequence is operatively linked to an expression control sequence to form an expression vector capable of expressing CYP27 and/or a CYP27 regulator. The transfected cells may be cells derived from the patient's normal tissue, the patient's diseased tissue, or may be non-patient cells. For example, blood vessel cells removed from a patient can be transfected with a vector capable of expressing CYP27 and/or a CYP27 regulator of the present invention, and be re-introduced into the patient. The transfected cells demonstrate modulated CYP27 expression and/or activity in the patient that modulate cholesterol efflux. Patients may be human or non-human animals. Cells may also be transfected by non-vector, or physical or chemical methods known in the art such as electroporation, incorporation, or via a "gene gun." Additionally, CYP27 and/or CYP27 regulator DNA may be directly injected, without the aid of a carrier, into a patient. In particular, CYP27 and/or CYP27 regulator DNA may be injected into blood.

The gene therapy protocol for transfecting CYP27 and/or a CYP27 regulator into a patient may either be through integration of CYP27 and/or CYP27 regulator DNA into the genome of the cells, into minichromosomes or as a separate replicating or non-replicating DNA construct in the cytoplasm or nucleoplasm of the cell. Modulation of CYP27 expression and/or activity may continue for a long-period of time or may be reinjected periodically to maintain a desired level of CYP27 expression and/or activity in the cell, the tissue or organ.

In a further preferred aspect of the present invention, there is provided a method of treating a cholesterol related condition in a patient by modulating cholesterol efflux in the patient, said method comprising:

introducing modulated cells to the patient, wherein said cells have modulated expression and/or activity of CYP27.

The modulated cells are intended to replace existing cells such that the existing cholesterol efflux of the cells is modulated or the modulated cells may be used to infiltrate existing regions of disease such as in an atheroma to halt progression of the atheroma. Preferably, the cholesterol efflux is increased by increasing expression and/or activity of CYP27. More preferably, the expression and/or activity of CYP27 is increased by transfection of a gene encoding CYP27 to the cells. The gene may then be over expressed or tuned on the increase expression of the CYP 27.

The replaced cells may be any cell that can efflux cholesterol and may be tissue specific for the condition to be treated. Preferably the cell is selected from the group including, but not limited to, hepatic cells, macrophages, vessel cells, endothelial cells or stem cells. Preferably the stem cells are capable of differentiating to any of the cells selected from the group including hepatic cells, macrophages, vessel cells, or endothelial cells. Preferably, the stem cells are hematopoietic stem cells. It is preferred that when using stem cells the cells may be transfected with a gene that encodes CYP27, such that upon implantation to a tissue requiring treatment, the cells may differentiate to the cells of the region. Regulators modulating sequences of a vector that has been introduced to the cell can switch expression of CYP27 on or off accordingly to the level of cholesterol efflux. These cells will eventually replace diseased cells that have accumulated cholesterol.

Preferably the cell that is targeted is directly related to the condition, such as but not limited to vessel cells which line the vessel and are directly related to conditions such as atherosclerosis.

Examples of the procedures used in the present invention will now be more fully described. It should be understood, however, that the following description is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

In yet another aspect of the present invention there is provided a method of identifying a compound which modulates cholesterol efflux in a cell, said method comprising:

contacting the compound to the cell;

detecting a change in CYP27 expression and/or activity in the cell relative to a cell which has not been contacted with the compound.

The present applicants have identified a target which provides an indication of cholesterol efflux in the cells. Cholesterol efflux is regulated by CYP27. Hence compounds which can modulate a change in CYP27 can influence cholesterol efflux.

Preferably the cell is a cell in which cholesterol efflux can be readily measured. Preferred cells include endothelial cells, smooth muscle cells and other cells of the vessel wall.

Measuring cholesterol efflux may be conducted by any means which can measure the movement of a compound such as cholesterol in a cell. Methods such as labelling the cholesterol with radioactive labels maybe utilised. However, these methods are familiar to the skilled addressee.

The contacting of the cell to the unknown compound may be by any means dependent upon the characteristics of the compound providing the cell can interact and be influenced by the compound to be tested. For instance, if the compound is soluble, this would involve direct contacts with the cell. However, if insoluble, suspensions of the compounds may be used.

These skills will be familiar to the person testing the compound.

The identification of the compound which can modulate cholesterol efflux will depend on its effect of changing expression and/or activity of CYP27 in the cell.

Preferably, the change increases expression and/or activity of CYP27 in the cell to identify a compound which increases cholesterol efflux. Alternatively, it is preferred that the change decreases expression and/or activity of CXP27 in the cell to decrease cholesterol efflux from the cell. Compounds may therefore be selected based on the effect on CYP27.

Methods of detecting the change in expression and/or activity of CYP27 may include measurement of the genetic expression of CYP27 including DNA, RNA, mRNA expression in the cell. Measurement of changes in these parameters in the cell are familiar to the skilled person.

Measurement of CYP27 by the use of antibodies may also be used to identify a change in CYP27 in the presence of absence of the unknown compound that is being tested.

Activity of CYP27 can be measured by the increase or decrease of oxidising cholesterol to convert cholesterol to 27 hydroxy cholesterol+3-beta-hydroxy-5-cholestenoic acid.

Once a compound is identified to change CYP27 expression and/or activity then it may be utilised in the manufacture of a medicament to treat cholesterol related conditions. Accordingly, the invention also provides a method of obtaining a composition when used for treating a cholesterol related condition, said method comprising:

providing a compound in an amount effective to treat the condition wherein said compound is identified by the methods described above; and

admixing the compound with a pharmaceutically acceptable carrier.

A pharmaceutically acceptable carrier may be any carrier known to the skilled addressee which is not toxic to the patient and which can be admixed to form a pharmaceutical.

Examples of the procedures used in the present invention will now be more fully described. It should be understood, however, that the following description is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

EXAMPLES

Example 1: Expression of sterol 27-hydroxylase (CYP27A1) enhances cholesterol efflux.

1. Methods

a) Cells.

CHOP–C4 cells were grown in RPMI 1640 medium containing 10% FBS, 2 mM L-glutamin, penicillin/streptomycin (50 units/ml) and 0.2 mg/ml G418. The day before transfection, cells were plated in 12 well plates at a density of 0.6 x105 cells per well. Transfection was performed with DEAE-Dextran as described in Albiston, A. L., Obeyesekere, V. R., Smith, R. E., and Krozowski, Z. S. (1994) *Mol. Cell. Endocrinol.* **105,** R11-17 using 200 ng of plasmid DNA (CYP27A1 in pcDNA1 or pcDNA1 alone) per well. CYP27A1 was isolated from a human kidney cDNA library during studies of progesterone metabolism using a screening protocol previously described for the isolation of 11ßHSD2 (Albiston, A. L *et al* (1994)).

b) Cholesterol Acceptors.

Blood from healthy normolipidemic volunteers was collected in saline containing streptokinase (Sigma, final concentration 150 units/ml) and plasma was isolated by repeated centrifugation for 15 min at 3000 rpm at 40C. Apolipoprotein A-I was isolated as described previously in Morrison, J. R., Fidge, N. H., and Grego, B. (1990) *Anal. Biochem.* **186,** 145-152.

c) Cholesterol efflux.

Two methods were used to label cellular cholesterol. Metabolic labeling was conducted by incubating cells in serum-free medium with [1-^{14}C] acetate (Amersham-Pharmacia-Biotech (APB), specific radioactivity 2.07 GBq/mmol,

final radioactivity 0.4 MBq/ml) for 24 h at 37°C in a CO_2 incubator. Alternatively, cells were incubated in serum containing medium with [1α,2α(n)-3H]cholesterol (APB, specific radioactivity 1.81 TBq/mmol, final radioactivity 0.2 MBq/ml) for 48h in a CO_2 incubator. After labeling, cells were washed six times with PBS and further incubated for 2h or the indicated periods of time at 37°C with serum-free medium containing either lipid-free apoA-I (final concentration 30μg/ml), or the indicated concentrations of human serum. The medium was then collected, centrifuged for 15 min at 40°C at 30,000g to remove cellular debris and the supernatant counted or used for further analysis. Cells were harvested using a cell scraper, dispensed in 0.5 ml distilled water and aliquots were counted or used for further analysis. Cholesterol efflux is expressed as a percentage of labeled cholesterol transferred from cells to the medium.

d) Lipid analysis.

Lipids were extracted with 3 volumes ethylacetate and separated using TLC (chloroform/ethylacetate 4:1 v/v) (Penning, T. M., Burczynski, M. E., Jez, J. M., Hung, C. F., Lin, H. K., Ma, H., Moore, M., Palackal, N., and Ratnam, K. (2000) *Biochem. J.* **351,** 67-77). TLC plates containing labeled lipids were exposed to a phosphoimager plate and analyzed on the Bioimager BAS-1000 (Fuji) and the radioactivity in each spot quantified. Spots of cholesterol and 27-hydroxycholesterol identified by standards (Research Plus, USA) were scraped and counted.

e) Northern blotting.

RNA was separated on a 1.2% agarose gel, transferred to a nylon membrane and probed with ^{32}P-labeled mouse ABCA1 cDNA (Chambenoit, O., Hamon, Y., Marguet, D., Rigneault, H., Rosseneu, M., and Chimini, G. (2001) *J. Biol. Chem.* **276,** 9955-9960) (gift of Dr. G. Chimini) and ^{32}P-labeled GAPDH cDNA as an internal standard. The membrane was exposed to a phosphoimager plate and analyzed on the Bioimager BAS-1000 (Fuji).

f) Statistical analysis.

The Student's *t*-test was used to determine statistically significant differences between groups.

2. Results

Transfection of CHOP cells with 27-hydroxylase (CYP271) resulted in production of 27-hydroxycholesterol when cells were metabolically labeled with [^{14}C] acetate. The amount of newly synthesized 27-hydroxycholesterol found in transfected cells was 5-fold of that in non-transfected cells, however it was only 1% of the amount of newly synthesized cholesterol found in transfected cells (Figure. 1). When transfected cells were exposed to plasma for two hours, 80% of the newly synthesized 27-hydroxycholesterol, (Figure. 1) and 45% of newly synthesized cholesterol (not shown) was released to plasma. The contribution of 27-hydroxycholesterol to the overall amount of sterol released to plasma was therefore 1.5%. Thus although 27-hydroxycholesterol was released from cells more readily than cholesterol, its contribution to overall sterol efflux was minimal. When the cellular cholesterol pool was labeled with [^{3}H] cholesterol, no formation of 27-hydroxycholesterol in either transfected or non-transfected cells was detected (not shown). To study the effect of transfection with CYP27 on cholesterol efflux, cellular cholesterol was labeled with [^{3}H] cholesterol and cells incubated in the presence or absence of whole human plasma, or lipid-free human apoA-I. Transfection of cells with CYP27 resulted in a 3-fold increase in cholesterol efflux to whole plasma, and a doubling of the efflux to apoA-I compared to both non-transfected and mock transfected cells (Figure. 2). Cholesterol efflux to the medium without acceptors was not affected by transfection. When lipids released to the medium were analyzed, no 27-hydroxycholesterol was found and all released radioactivity was accounted for in the cholesterol fraction (not shown). The time-course of cholesterol efflux to human plasma is shown in Figure 3A. The kinetics consists of "fast" and "slow" phases as reported by Gaus, K., Gooding, J. J., Dean, R. T., Kritharides, L., and Jessup, W. (2001) *Biochemistry* **40,** 9363-9373). Transfection of cells with CYP27 resulted in a significant stimulation of the "fast" phase of the efflux with modest stimulation of the "slow" phase. Dose-dependence of the efflux is shown in Figure 3B. Relatively more cholesterol was released from transfected cells at low plasma concentration, and the difference between transfected and non-transfected cells gradually disappeared when concentration of plasma increased. 27-hydroxycholesterol is a ligand of LXR receptor regulating a

number of genes involved in lipid metabolism including ABCA1 (Fu, X., Menke, J. G., Chen, Y., Zhou, G., MacNaul, K. L., Wright, S. D., Sparrow, C. P., and Lund, E. G. (2001) *J. Biol. Chem.* **276,** 38378-38387). To investigate if formation of 27-hydroxycholesterol lead to changes in the expression of ABCA1, the expression of ABCA1 in transfected and non-transfected cells was evaluated. ABCA1 was identified on the Northern blot using mouse ABCA1 cDNA probe as a single band (not shown). There was little difference in the abundance of ABCA1 mRNA between transfected and mock-transfected cells. The ratio of ABCA1 to GAPH mRNA was 0.84 and 1.36 for transfected and mock-transfected cells, respectively.

The major finding is that transfection of cells with sterol 27-hydroxylase stimulates cholesterol efflux. The stimulation of cholesterol efflux could be explained by two mechanisms. Without being limited by this hypothesis, firstly, appearance of hydroxycholesterol in the plasma membrane may change the properties of the membrane and facilitate the release of cholesterol and/or 27-hydroxycholesterol. Secondly, since 27-hydroxycholesterol is a ligand of the LXR receptor which regulates a number of genes involved in cholesterol homeostasis, in particular ABCA1 (Fu, X *etal* (2001)), 27-hydroxycholesterol may enhance cholesterol efflux via effects on these genes. Several of these findings point to the likelihood of the first mechanism. Firstly, there was no induction of ABCA1 in transfected cells. Secondly, the effect of transfection was more pronounced with whole plasma than with lipid free apoA-I, the opposite would be expected if the enhanced efflux resulted from stimulation of the expression of ABCA1. Furthermore transfection mainly stimulated the "rapid" phase of the efflux, i.e. release of cholesterol already present in the plasma membrane (Gaus, K *etal* (2001)) making involvement of pathways required for mobilization of intracellular cholesterol unlikely. Transfection of cells with CYP27 resulted in the production of 27-hydroxycholesterol when sterols were labeled metabolically, but not when the cholesterol pool was labeled with [^{3}H] cholesterol. This may be due to the inability of exogenous cholesterol to access the mitochondrial enzyme (Cali, J. J., and Russell, D. W. (1991) *J. Biol. Chem.* **266,** 7774-7778). Even with metabolic labeling the proportion of newly synthesized 27-hydroxycholesterol was low and increased efflux was found to

be mainly if not entirely due to stimulation of the efflux of non-oxidized cholesterol.

The discussion of documents, acts, materials, devices, articles and the like is included in this specification solely for the purpose of providing a context for the present invention. It is not suggested or represented that any or all of these matters formed part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia before the priority date of each claim of this application.

Finally it is to be understood that various other modifications and/or alterations may be made without departing from the spirit of the present invention as outlined herein.

DATED: 24 September, 2002
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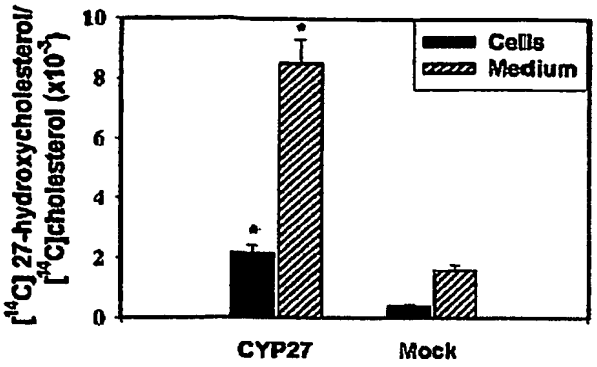
[14C] 27-hydroxycholesterol/
[14C]cholesterol (x10-3)
10
8
6
4
2
0
Cells
Medium
CYP27
Mock

Fig. 1

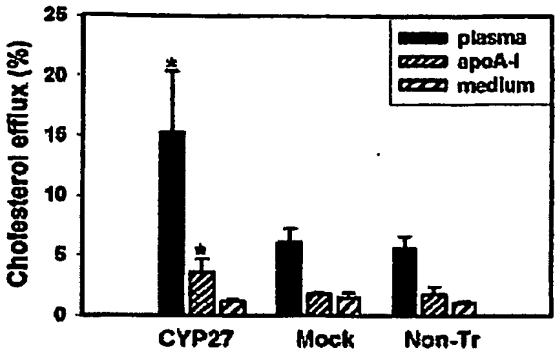
Cholesterol efflux (%)
25
20
15
10
5
0
plasma
apoA-I
medium
*
*
CYP27
Mock
Non-Tr

Fig. 2

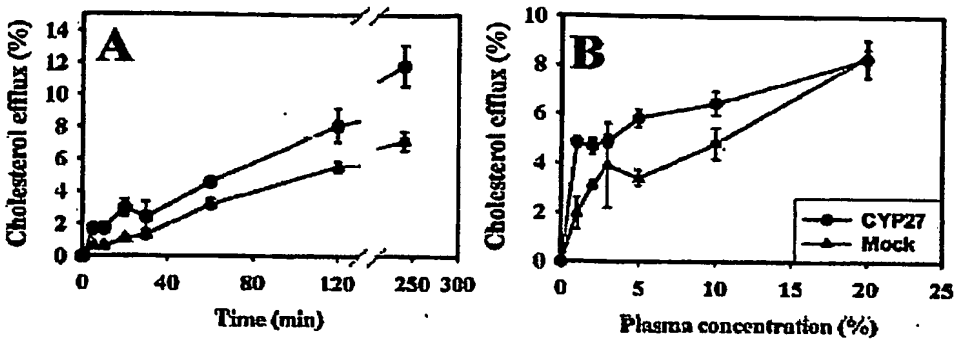
A
Cholesterol efflux (%)
14
12
10
8
6
4
2
0
0
40
80
120
250
300
Time (min)
B
Cholesterol efflux (%)
10
8
6
4
2
0
0
5
10
15
20
25
Plasma concentration (%)
CYP27
Mock

Fig. 3